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Ikaros Is a Negative Regulator of B1 Cell Development and Function*

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Alejandra Macias-Garcia[‡1,2], Beate Heizmann[‡2,3], MacLean Sellars[‡4], Patricia Marchal[‡], Hayet Dali[§], Jean-Louis Pasquali[‡§¶], Sylviane Muller[§], Philippe Kastner[‡ ‡‡5], and Susan Chan[‡5]**

*From the [‡]Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), INSERM U964, CNRS UMR 7104, Université de Strasbourg, 67404 Illkirch, France, [§]Institut de Biologie Moléculaire et Cellulaire (IBMC), CNRS UPR3572, 67000 Strasbourg, France, [¶]UFR Médecine, Université de Strasbourg, 67000 Strasbourg, France, [‖]Department of Clinical Immunology, Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France, **Institut d'Etudes Avancées, Université de Strasbourg, 67000 Strasbourg, France, and [‡‡]Faculté de Médecine, Université de Strasbourg, 67000 Strasbourg, France*

B1 B cells secrete most of the circulating natural antibodies and are considered key effector cells of the innate immune response. However, B1 cell-associated antibodies often cross-react with self-antigens, which leads to autoimmunity, and B1 cells have been implicated in cancer. How B1 cell activity is regulated remains unclear. We show that the Ikaros transcription factor is a major negative regulator of B1 cell development and function. Using conditional knock-out mouse models to delete Ikaros at different locations, we show that Ikaros-deficient mice exhibit specific and significant increases in splenic and bone marrow B1 cell numbers, and that the B1 progenitor cell pool is increased ~10-fold in the bone marrow. Ikaros-null B1 cells resemble WT B1 cells at the molecular and cellular levels, but show a down-regulation of signaling components important for inhibiting proliferation and immunoglobulin production. Ikaros-null B1 cells hyper-react to TLR4 stimulation and secrete high amounts of IgM autoantibodies. These results indicate that Ikaros is required to limit B1 cell homeostasis in the adult.

B1 B cells are an important part of the innate immune response because they spontaneously secrete natural, polyreactive IgM antibodies in the absence of infection (1, 2). In the mouse, they differ from conventional B2 B cells (follicular and marginal zone) in that they do not express CD21 and CD23, and express only low levels of B220 and IgD but high levels of IgM. B1 cells are abundant in the peritoneal and pleural cavities where they can be further divided into B1a ($CD19^+CD5^+CD11b^+$) and B1b ($CD19^+CD5^-CD11b^+$) cells. Splenic B1 cells are $CD19^+CD43^+CD5^+$. The majority of B1 cells develop early *in utero* from fetal liver progenitor cells, and they are maintained over time in the adult through self-renewal. However, $Lin^-CD93^+CD19^+B220^{lo}$ bone marrow B1 progenitor cells have been identified (3). In addition, B cell receptor (BCR)[6] signal strength appears to be important for B1 cell generation, as strong signals increase B1 cell numbers and weak signals decrease their numbers (4, 5).

Because natural antibodies are polyreactive, they also bind to self-antigens and contribute to autoimmunity, suggesting that B1 cells must be tightly regulated during homeostasis. In addition, because they comprise the first wave of B cell development, B1 cells may be linked to childhood leukemias. Work in recent years have begun to reveal a network of transcriptional regulators important for B1 cell development and function. Among them, members of the classical NFκB pathway (*e.g.* p50, Malt1, Carma1, Ikk complex), downstream of the BCR, have been shown to be essential for B1 cell development (6). The RNA-binding protein Lin28b, and its downstream effectors Let-7 and Arid3a, were revealed to promote fetal B1 cell lymphopoiesis (7, 8). Similarly, Ebf1 is required, and its overexpression induces B1 cell development at the expense of B2 cells (9, 10). In contrast, PU.1 (encoded by *Spi1*) has been reported to keep B2 cells from "switching" to the B1 cell lineage (11), and Pax5 and E2a (encoded by *Tcf3*) have been shown to be important for B2 cell identity (12, 13). Blimp1 (encoded by *Prmd1*) and Xbp1 are required for B1 (and B2) cell immunoglobulin secretion (14), while Irf4 is required for IgM secretion by splenic B1 cells (15). Interestingly, NFκB, Lin28b/Let-7/Arid3a, Ebf1, Blimp1, and Xbp1 all work to positively regulate B1 cell development and function. Regulators that limit B1 cell activity are still poorly understood.

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[1] Received predoctoral fellowships from the Conacyt Association of Mexico and the Fondation ARC. Present address: Institute for Medical Engineering and Science at MIT, Cambridge, MA 02139.

[2] Both authors contributed equally to this work.

[3] Received post-doctoral fellowships from the Fondation ARC and Fondation de France. To whom correspondence may be addressed: IGBMC, 1 rue Laurent Fries, 67404 Illkirch Cedex, France. Tel.: 33-3-88-65-34-61; Fax: 33-3-88-65-32-01; E-mail: heizmann@igbmc.fr.

[4] Received a predoctoral fellowship from the Fondation pour la Recherche Médicale. Present address: David Geffen School of Medicine at UCLA, Los Angeles, CA 90095.

[5] To whom correspondence may be addressed. E-mail: scpk@igbmc.fr.

[6] The abbreviations used are: BCR, B cell receptor; T-ALL, T cell acute lymphoblastic lymphomas/leukemias; BM, bone marrow; FO, follicular; MZ, marginal zone; PEC, peritoneal cavity; TAM, tamoxifen; 4OHT, 4-hydroxytamoxifen; B-ALL, B cell acute lymphoblastic leukemias.

Ikaros, encoded by the *Ikzf1* gene, is a zinc finger DNA-binding protein, that is a key transcriptional regulator and tumor suppressor in B cells. It is required for the specification and development of all B cell lineages (16, 17), and plays specific roles in pre-pro-B and pre-B cells to activate *RAG1,2* expression, mediate chromatin accessibility during immunoglobulin gene rearrangement and allelic exclusion at the *Igk* locus (18–23). In mature B2 cells, Ikaros directs *Ig* class switch recombination (24). It functions both as a transcriptional repressor and activator, and acts at least in part through its association with Polycomb repressive complex 2 (25), NuRD and SWI/SNF complexes (26, 27). In pre-B cells, Ikaros activates the transcription of genes important for pre-BCR and BCR signaling, cell survival, and cell migration, as well as that of B cell regulators like *Pax5*, *Foxo1*, and *Ebf1* (22, 28). Thus Ikaros modulates B cell function at multiple stages. Here, we reveal a novel function for Ikaros as a major negative regulator of B1 cell development and function in the adult bone marrow and spleen.

Experimental Procedures

Mice—The $Ik^{L/L}$ and $Ik^{f/f}$ mouse lines have been described (18, 22). $Ik^{L/L}$ mice were backcrossed 10 generations onto the C57Bl/6 background and analyzed at 6–8 weeks of age. $Ik^{f/f}$ mice were crossed with CD21-Cre, CD19-Cre, or R26-CreERT2 tg animals (29–31). Ikaros was deleted in adult $Ik^{f/f}$ R26-CreERT2^{+} mice after daily intraperitoneal injections of tamoxifen (50 mg/kg weight of mouse, dissolved in sunflower oil) for 3 days. Female MRL/lpr mice were purchased from Harlan.

Cell Culture—FO B cells were sorted ($B220^{+}CD23^{hi}CD21^{lo}$; >98% purity) on a FACSVantage S.E. option DiVa (BD Biosciences, San Jose, CA) or a FACSAria II SORP (BD Biosciences), or enriched by depletion of $CD43^{+}$ cells followed by positive selection of $CD23^{+}$ cells with MACS beads (>90% purity; Miltenyi Biotech, Bergisch Gladbach, Germany). Both methods gave similar results. B1 B cells were sorted ($CD19^{+}CD43^{+}$) on a FACSAria II SORP (BD Biosciences). For BM cultures, 1×10^6 $CD19^{+}$ BM B cells were co-cultured on S17 stromal cells in Iscove's medium supplemented with 10% FCS, 2 mM L-glutamine, 1× non-essential amino acids, 50 μM 2-mercaptoethanol (2-ME), 1% antibiotics plus cytokines IL-7 (7% of supernatant from mIL-7 cDNA-transfected J558L cells), SCF (10 ng/ml; Peprotech, Rocky Hill, NJ) and Flt-3 ligand (2.5% of supernatant from mFlt3L cDNA-transfected B16 cells). For proliferation assays, cells were labeled with CFSE (5 μg/ml; Sigma) and 2.5–3×10^4 cells were cultured in complete medium (RPMI 1640, 10% FCS, 25 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 1× non-essential amino acids, 50 μM 2-ME, 1% antibiotics). Cells were stimulated with 10 μg/ml goat anti-mouse IgM F(ab′)2 (Jackson ImmunoResearch, West Grove, PA), 2–10 μg/ml mouse anti-CD40 (eBiosience, San Diego, CA), 10 ng/ml IL-4 (Peprotech), and 25 μg/ml LPS (*Escherichia coli* 0111:B4; Sigma). For intracellular staining cells were fixed with 2% paraformaldehyde (PFA) and permeabilized with methanol. For cell viability assays, $DiOC_6$ (Molecular Probes, Eugene, OR) was added at 50 nM. For cell cycle staining, PFA fixed cells were permeabilized with 0.1% saponin, and stained with anti-Ki67-AF700 (BD Biosciences) and DAPI (2 μg/ml).

Flow Cytometry—The following reagents were used: anti-B220-PE-Cy7, anti-CD19-PerCP-Cy5.5, anti-CD21-APC or -FITC, anti-CD23-PE-Cy7, anti-CD43-FITC, -PECy7 or -PE, anti-IgD-FITC, anti-CD11b-APC, anti-TCR$\alpha\beta$-biotin, anti-TCR$\gamma\delta$-biotin, and streptavidin (SA)-APC-Cy7 from BD Biosciences; anti-CD16/32, anti-CD23-biotin, anti-CD3ϵ-biotin, anti-CD71-biotin, anti-Gr-1-biotin, anti-NK1.1-biotin, anti-CD8-biotin, anti-CD5-biotin, anti-CD5-FITC, and anti-CD93-APC from eBioscience; anti-CD59-biotin from BMA Biomedical (Augst, Switzerland); anti-IgM-PE, -FITC or -APC, and SA-PE from Jackson ImmunoResearch; anti-rabbit IgG (H+L)-biotin from Vector Laboratories (Burlingame, CA); SA-Alexa Fluor 405 and anti-rat IgG-Qdot 605 from Invitrogen (Carlsbad, CA). Lin^{-} cells were defined by staining with a mixture of antibodies for mature hematopoietic lineage marker including CD3ϵ, CD11b, CD59, CD71, Gr-1, NK1.1, TCR$\alpha\beta$, TCR$\gamma\delta$, CD8, and IgM. Cells were analyzed with a FACSCalibur and FACS LSR II (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

ELISA—ELISAs of sera were performed as described (33). Briefly, polystyrene plates (MaxiSorb; Nunc, Rochester, NY) were coated ON at 37 °C with the following antigens: dsDNA (100 ng/ml in 25 mM citrate buffer, pH 5.4; Sigma) and mouse chromatin extracted and purified from mouse lymphocytic leukemia cells L1210 (200 ng/ml expressed as dsDNA concentration in PBS, pH 7.2). Mouse sera (diluted 1:250 in PBS containing 0.05% Tween-20 (T) and 1% BSA) were added for 1 h, followed by goat anti-mouse IgG (diluted $1{:}2 \times 10^4$ in PBS-T; Fcγ specific; Jackson ImmunoResearch) supplemented with goat anti-mouse IgG3 (diluted 1:7,500; Fcγ specific; Nordic Immunology, Tilburg, Netherlands) or by goat anti-mouse IgM (diluted $1{:}2 \times 10^4$; μ chain specific F(ab′)2; Jackson ImmunoResearch) conjugated to horseradish peroxidase. After a 30-min incubation at 37 °C, H_2O_2 and 3,3′,5,5′-tetramethyl benzidine were added as substrate and chromogen, respectively, for 15 min. The reaction was stopped, and the absorbance was measured at 450 nm.

For ELISAs of cell culture supernatants, polystyrene plates (MaxiSorb; Nunc) were coated overnight at 4 °C with the following antigens: salmon dsDNA (100 μg/ml in PBS, pH 7.2; Sigma) or mouse chromatin (15 μg/ml) extracted and purified from total mouse splenocytes as described (34). Plates were blocked with 3% BSA in PBS containing 0.05% Tween-20 1h at 37 °C. Supernatants (undiluted) or mouse sera (diluted 1:250 in PBS containing 0.05% Tween-20 and 1% BSA) were added for 2 h at room temperature followed by goat anti-mouse IgM (diluted $1{:}2 \times 10^4$; μ chain specific F(ab′)2; Jackson ImmunoResearch) conjugated to alkaline phosphatase. After a 2-h incubation at room temperature, *p*-nitrophenyl phosphate was added as substrate for 20 min and the absorbance measured at 405 nm.

Detection of Antinuclear Antibodies (ANAs)—Assays were performed by indirect immunofluorescence with HEp-2 cells (HEp-2 substrate slides; Zeus Scientific, Raritan, NJ). HEp-2 cell-coated slides were incubated for 30 min at room temperature with diluted sera, washed 2× in PBS and visualized by fluorescence microscopy with FITC-labeled anti-mouse IgG or

IgM (1:100). Controls included negative and positive sera from BALB/c and MRL/lpr mice, respectively.

Western Blot—Whole cell lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). The following antibodies were used: anti-Ikaros (rabbit polyclonal antibody generated against the C terminus of Ikaros; in-house), anti-β-actin (Sigma), anti-rabbit, and anti-mouse HRP (Jackson Immunoresearch). Blots were revealed with Immobilon Western Chemiluminescent HRP substrate (Millipore).

RT-qPCR and Primers—RNA was extracted using RNeasy kit (Qiagen, Hombrechtikon, Switzerland). RNA was reverse described with Superscript Reverse Transcriptase (Invitrogen). RT-qPCR was performed with 1 cycle at 95 °C for 15 min, then 50 cycles of 95 °C for 10 s, 60 °C or 63 °C for 10 s and 72 °C for 15 s with SYBR Green PCR MasterMix (Sigma or Roche) on an LC480 light cycler (Roche, Basel, Switzerland). The following primers were used: Irf4 (5′-AAGGCAAGTT CCGAGAAGGG, 5′-TTATGAACCTGCTGGGCT GG), Pax5 (5′-CAGATGT-AGTCCGCCAAAGGATAG, 5′-ATGC CACTGATGGAGT-ATGAGGAG CC), Tcf3 (5′-ATACAGCGAAGGTGCCC-ACT, 5′-CTCAAGGT GCCAACACTGGT), Ebf1 (5′-CTA TGTGCGC CTCATCGACT, 5′-CATGATCTCGT GTG-TGAG CAA), Rapgef4 (5′-CAAGGAGAATGTCCCTTC AG-AGA, 5′-CCGCGAGTGAACACAGGAT), Nrp2 (5′-GACTT-CATTGAGATTCGGGATGG, 5′-AACTTGATGTATAAC-ACGGAGCC), Tmem176b (5′-ACTGTCCGGCCAGGTAT-CAT, 5′-TGCCTGATTAAGCTGTTCACAC), Gna15 (5′-AAGAGCGCGAGGAATTGAAAC, 5′-GAGTA GCCCACA-CCGTGAATG), Slamf9 (5′-TTCAA AACAACATTGCCAT-CGTG, 5′-CCCAGGTCAG ATTGCTAATATGC), Bcl2 (5′-GTCGCTACCGT CGTGACTTC, 5′-CAGACATGCACCTA-CCCA GC), Bcl2l1 (5′-GACAAGGAGATGCAGGTATT GG, 5′-TCCCGTAGAGATCCACAAAAGT). The primers for Spi1 (11), Prmd1 and Xbp1 (14), Slpi (35), Tyrobp (36), Pirb (37), Mcl1 (38), Ikzf1, and Hprt (22) were used as described.

Microarray Analysis—Total RNA was extracted with the RNeasy Micro kit (Qiagen). RNA quality was verified with a 2100 Bioanalyzer (Agilent, Santa Clara, CA). Biotinylated single strand cDNA targets were prepared from 150 ng of total RNA with the Ambion WT Expression Kit and the Affymetrix GeneChip® WT Terminal Labeling Kit. After fragmentation and end-labeling, 1.9 μg of cDNAs were hybridized to Affymetrix GeneChip® Mouse Gene 1.0 ST arrays using standard procedures. Raw data were processed with the Robust Multiarray Average (RMA) algorithm. Microarray data are available in the GEO Data Bank (GSE 75827).

Chromatin Immunoprecipitation and ChIP-Sequencing—The ChIP protocol was adapted from the Millipore ChIP Assay Kit (17–295) with minor modifications and described (25). Shortly, BH1-Ik1-ER-Bcl2 pre-B cells (22) were treated with 4OHT for 24 h in the absence of IL-7 to induce Ikaros function. After crosslinking, the chromatin was sonicated to 300–500 bp using a Bioruptor 200 (Diagenode, Denville, NJ) and cleared by centrifugation. After pre-cleaning with protein A-Sepharose, the lysate was incubated overnight with the anti-Ikaros antibody. Precipitation was carried out using protein A-magnetic beads. De-crosslinked DNA was purified using the iPure Kit (Diagenode). ChIP-sequencing was performed as described (25).

Results

Ikaros Deficiency Alters B Cell Homeostasis in the Spleen—To evaluate mature B cell populations *in vivo*, we first analyzed $Ik^{L/L}$ mice carrying a germline hypomorphic *Ikzf1* mutation. $Ik^{L/L}$ cells express functional Ikaros proteins at ~10% of levels detected in WT cells (18, 39). Although $Ik^{L/L}$ mice die from T cell-acute lymphoblastic lymphomas/leukemias (T-ALL) at 4–6 months of age, the animals used here (6–8 weeks old) showed no signs of transformation in the thymus, as defined by CD4 and CD8 profiling, T cell receptor Vα and Vβ chain usage, and the absence of a deregulated Notch pathway (39, 40). In addition, mice with B cell-specific null mutations for *Ikzf1* were analyzed (22). $Ik^{f/f}$ mice were crossed with CD19-Cre tg animals to activate the Cre recombinase from the bone marrow (BM) pro-B cell stage, and with CD21-Cre tg mice to activate Cre activity from the splenic T2 B cell stage (29, 30, 32). Deletion of the floxed *Ikzf1* alleles at these stages did not grossly affect B cell differentiation in the BM, although $Ik^{f/f}$ CD19-Cre^{+} (CD19 cKO) mice showed an increase in pro- and large pre-B cells and a corresponding decrease in $B220^{+}CD43^{-}$ cells, suggesting a bottleneck in differentiation, in line with the requirement for Ikaros in pre-B cell development (Fig. 1*A*) (22). In the spleen, Ikaros proteins were detected in WT B cell populations (Fig. 1*B*) (22), but they were selectively absent in splenic B cells of both CD19 cKO and $Ik^{f/f}$ CD21-Cre^{+} (CD21 cKO) mice (Fig. 1*C*).

Splenic B cell populations were analyzed in the different mutant mice by flow cytometry. While total splenocyte numbers were reduced in $Ik^{L/L}$ mice, as previously described (18), they were slightly increased or similar in CD19 and CD21 cKO animals, respectively, compared with control mice (Fig. 1*D*). $CD19^{+}$ B cells were also reduced in the $Ik^{L/L}$ spleens but not in the cKO organs, suggesting that mature B cell numbers in general are not directly affected by Ikaros loss (Fig. 1*D*). Nonetheless, some B cell subsets were altered (Fig. 1, *E* and *F*). $Ik^{L/L}$ spleens contained less $CD19^{+}CD21^{lo}CD23^{hi}$ follicular (FO) but more $CD19^{+}CD21^{hi}CD23^{lo}$ marginal zone (MZ) B cells, while cKO spleens contained variable numbers of FO but no detectable MZ B cells, when compared with control organs. These results suggested that Ikaros loss in late B cell development does not significantly affect FO B cell homeostasis, whereas MZ B cell homeostasis depends on Ikaros function.

Unexpectedly, $CD19^{+}B220^{lo}CD43^{+}CD5^{+}IgM^{hi}IgD^{lo}$ B1 cells were significantly increased in frequency and absolute number in all mutant spleens (Fig. 2*A* and not shown). While control spleens contained 1–2% of B1 cells, as reported (41), $Ik^{L/L}$ and cKO spleens showed 2–4-fold more B1 cells. Similar increases were not observed in the peritoneal cavity (PEC), as both $CD19^{+}CD5^{+}CD11b^{+}$ B1a and $CD19^{+}CD5^{-}CD11b^{+}$ B1b cells were detected in similar or slightly reduced numbers in the mutant mice compared with control littermates (Fig. 2*B*). These results suggested that the splenic B1 cell compartment is consistently and selectively enhanced in the absence of Ikaros.

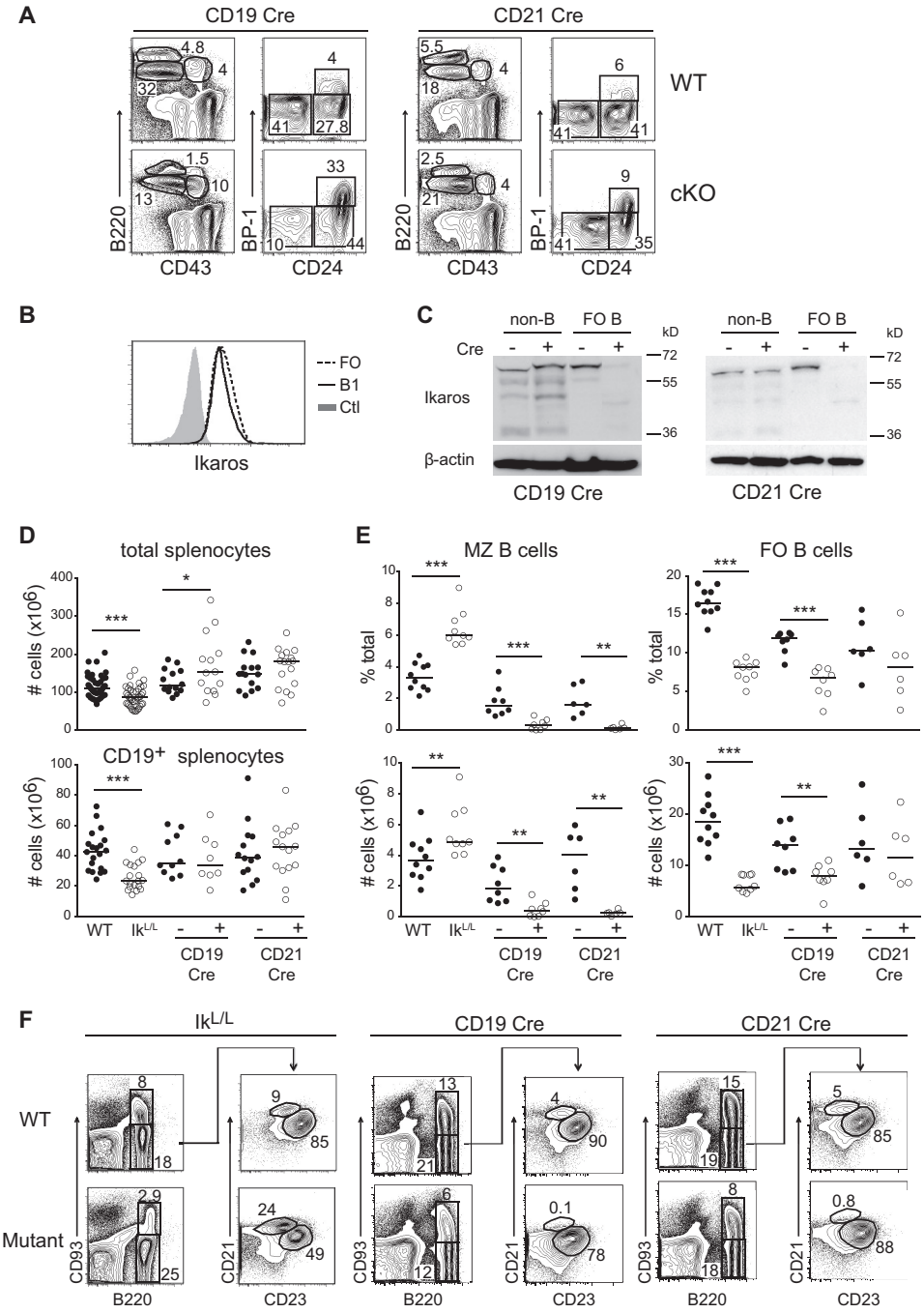


FIGURE 1. **Consequence of Ikaros deletion on bone marrow and splenic B cell populations.** *A*, BM B cell populations of the indicated mice. Gated populations in the *left panels*: pro- and large pre-B ($B220^+CD43^+$); small pre-B and immature B ($B220^+CD43^-$); recirculating B ($B220^{hi}CD43^-$). $B220^+CD43^+$ cells were further analyzed for CD24 and BP-1 in the *right panels*: pre-pro-B ($CD24^-BP\text{-}1^-$), early pro-B ($CD24^+BP\text{-}1^-$), late pro-B and large pre-B ($CD24^+BP\text{-}1^+$). Numbers correspond to percentages. *B*, Ikaros expression in FO and B1 cells was assessed by intracellular staining. As a control, the Ikaros-deficient cell line BH1 was used (*gray histogram*). *C*, Western blot of Ikaros proteins in $CD43^+$ non-B cells and FO B cells from spleens of the indicated mice. β-Actin was used as a loading control. The different Ikaros bands correspond to various isoforms. All data are representative of three independent experiments. *D*, graphs show cellularity (*top*), and absolute numbers of $CD19^+$ B cells (*bottom*) from the indicated mice. *Lines* denote median. Each *circle* represents one mouse, cumulative data from >5 independent experiments. *E*, graphs show percentages and absolute numbers of MZ and FO B cells from the indicated mice. *Lines* denote median. Each *circle* represents one mouse, cumulative data from >5 independent experiments. *F*, representative staining of splenic B cell populations of the indicated mice. $B220^+CD93^-$ mature B cells were analyzed for CD21 and CD23 expression to delineate FO ($CD23^{hi}CD21^{lo}$) and MZ ($CD23^{lo}CD21^{hi}$) B cells. *, $p < 0.01$; **, $p < 0.005$; ***, $p < 0.001$ (unpaired Student's *t* test).

Ikaros-null B1 Cells Develop Rapidly from Adult Progenitor Cells—Because B1 cells differentiate from fetal liver and BM progenitors (42), we asked if loss of Ikaros could affect B1 cell homeostasis in the adult bone marrow. The percentage and number of phenotypically mature $CD19^+IgM^{hi}CD43^+CD5^+$ B1 cells were therefore assessed in the BM of $Ik^{L/L}$, CD19 cKO and CD21 cKO mice (Fig. 3*A*). B1 cells were detected in significantly higher numbers in the $Ik^{L/L}$ and CD19, but not CD21, cKO BM compared with control samples, suggesting that Ikaros already plays a role in expanding BM B1 progenitor cells.

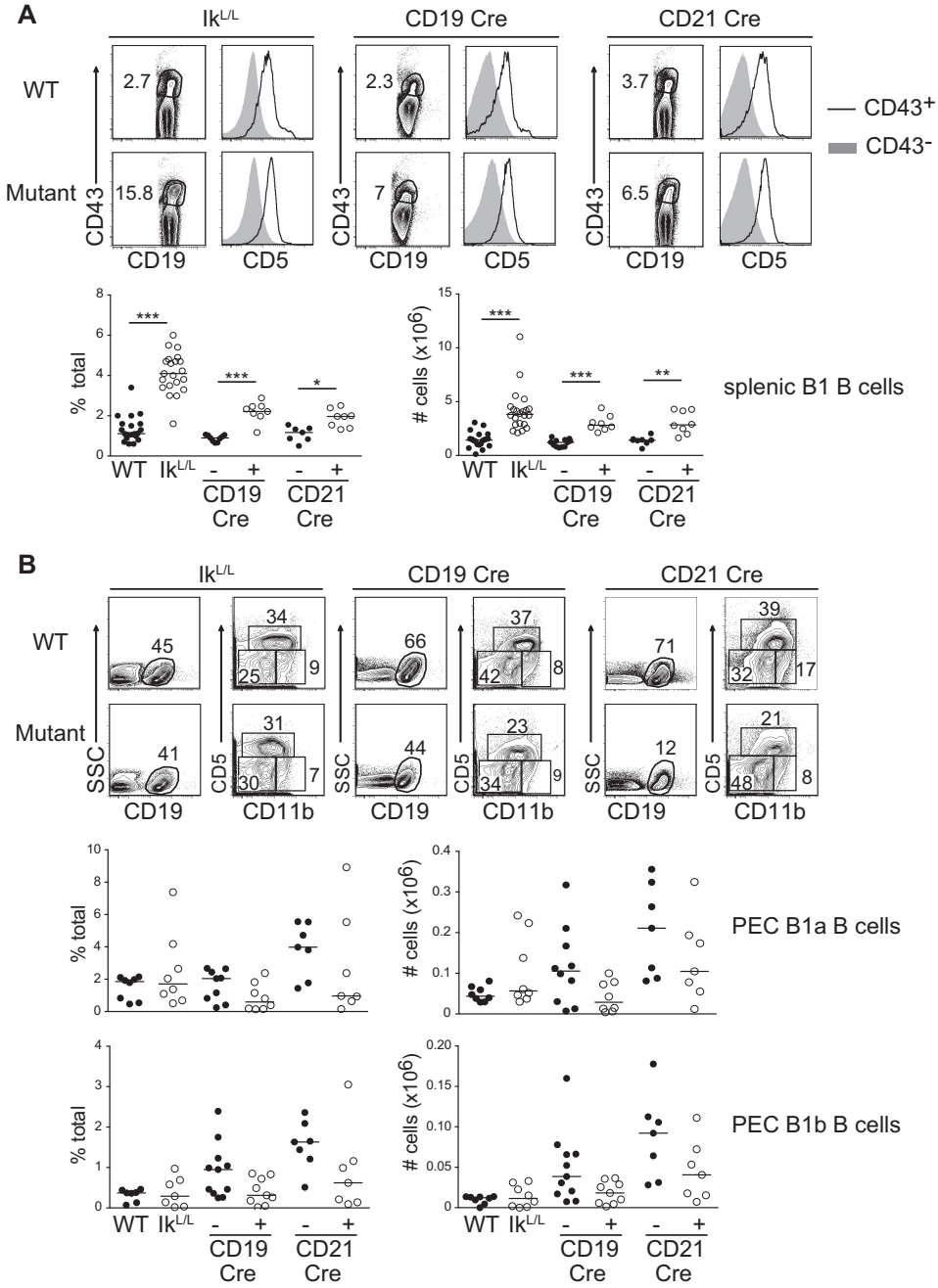


FIGURE 2. **Loss of Ikaros leads to accumulation of splenic B1 cells.** *A* (*top*) $CD19^+$ splenic B cells from the $Ik^{L/L}$, CD19 cKO, and CD21cKO mice were analyzed for CD43 expression by flow cytometry to define B1 cells ($CD19^+CD43^+$). Numbers correspond to percentages. $CD19^+CD43^+$ and $CD19^+CD43^-$ cells were further analyzed for CD5 expression. (*bottom*) Graphs show percentages and absolute numbers of splenic B1 cells in the indicated mice from >5 independent experiments. Each *circle* represents one mouse. *Lines* denote median. *B* (*top*) PEC $CD19^+$ cells were analyzed for CD11b and CD5 expression to delineate B1a ($CD11b^+CD5^+$) and B1b ($CD11b^+CD5^-$) cells. (*Bottom*) graphs show percentages and absolute numbers of PEC B1 cells from >5 independent experiments. Each *circle* represents one mouse. *Lines* denote median. *, $p < 0.003$; **, $p < 0.001$; ***, $p < 0.0001$ (unpaired Student's *t* test).

To determine if B1 progenitor cells are affected by Ikaros deficiency, we evaluated the number of $Lin^-CD93^+CD19^+B220^{lo}$ BM cells, previously shown to contain B1 progenitors (3), in $Ik^{L/L}$ and WT mice (Fig. 3*B*). This showed that the $Ik^{L/L}$ BM had significantly more $Lin^-CD93^+CD19^+B220^{lo}$ cells than the WT BM. These results thus suggested that the BM contains more B1 progenitor and mature cells when Ikaros function is impaired.

To determine if loss of Ikaros enhances B1 cell development throughout adult life, $Ik^{f/f}$ mice were crossed with R26-CreERT2 tg animals (31), and $Ik^{f/f}$ R26-$CreERT2^+$ (R26 cKO) animals and control littermates ($Ik^{f/f}$ R26-$CreERT2^-$) were analyzed after tamoxifen (TAM) injection to delete the $Ik^{f/f}$ alleles in all tissues. Mice were treated with TAM for 3 days, and loss of Ikaros proteins in splenocytes was confirmed 1 month later by Western blot (Fig. 3*C*). TAM treatment increased splenic $CD19^+CD43^+$ B1 cell numbers by ~3-fold in R26 cKO mice (Fig. 3*D*), while the numbers of $CD19^+CD43^-$ B cells (which includes transitional, FO and MZ B cells) decreased. These

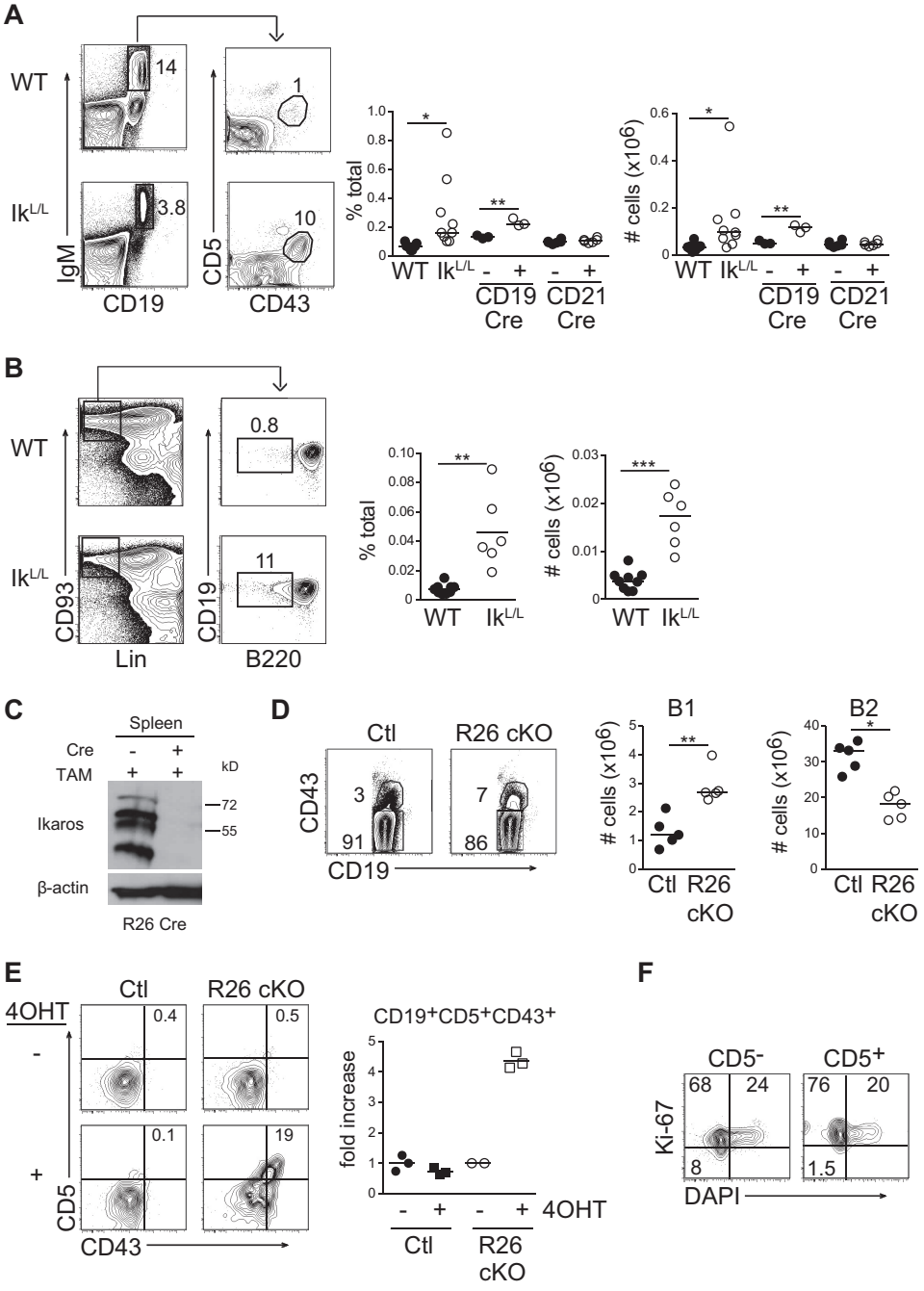


FIGURE 3. **Ikaros controls B1 cell differentiation in the bone marrow.** *A*, B1 cells ($IgM^+CD19^+CD5^+CD43^+$) in the WT and $Ik^{L/L}$ BM. Graphs show percentages and absolute numbers (one tibia and one femur) of BM B1 cells in the indicated mice from >5 independent experiments. Each *circle* represents one mouse. *Lines* denote median. *, $p = 0.01$; **, $p < 0.001$ (unpaired Student's *t* test). *B*, $Lin^-CD93^+CD19^+B220^{lo}$ B1 cell progenitors in the WT and $Ik^{L/L}$ BM. Graphs show percentages and absolute numbers (one tibia and one femur) of BM B1 cell progenitors from >5 independent experiments. Each *circle* represents one mouse. *Lines* denote median. **, $p < 0.005$; ***, $p < 0.0001$ (unpaired Student's *t* test). *C*, Western blot of Ikaros proteins in splenocytes of R26 cKO and control mice treated with TAM for 3 days and analyzed after 4 weeks. β-Actin was used as a loading control. The different Ikaros bands correspond to various isoforms. Representative of three independent experiments. *D*, mice were treated with TAM for 3 days. $CD19^+$ spleen cells were analyzed 1 month later for B1 ($CD19^+CD43^+$) and B2 ($CD19^+CD43^-$) cells. Graphs show absolute numbers from five independent experiments. *Lines* denote median. *Ctl*, control. *, $p < 0.01$; **, $p < 0.003$ (paired Student's *t* test). *E*, $CD19^+$ BM B cells were cultured on stromal cells and cytokines ± 4OHT. After 6 days, $CD19^+$ cells were analyzed for CD5 and CD43 expression. *Ctl*, control. The relative fold-increase in cell numbers of $CD19^+CD5^+CD43^+$ cells is shown where control values were set at 1.0, from three independent experiments. *F*, $CD19^+CD5^+$ or $CD19^+CD5^-$ cells from the R26 cKO + 4OHT cell cultures (see *E*) were analyzed by Ki-67 and DAPI staining. Representative staining from three independent experiments.

results indicated that splenic B1 cell numbers are rapidly increased in adult mice when Ikaros function is lost.

To demonstrate that B1 cells develop from BM progenitor cells in the absence of Ikaros, we co-cultured R26 cKO or control $CD19^+$ BM cells with S17 stromal cells and cytokines for 6 days under B lymphopoietic conditions (11), in the presence or absence of 4-hydroxytamoxifen (4OHT) to induce *Ikzf1* deletion. These experiments showed that $CD19^+CD43^+CD5^+CD11b^-$ cells were present in significant numbers in the 4OHT-treated R26 cKO cultures, but not in the control wells (Fig. 3*E* and not shown). Further, these cells were similar to $CD19^+CD5^-$ non-B1 B cells in cell cycle status (Fig. 3*F*), suggesting that they

appeared as a result of differentiation and not increased proliferation. Altogether, our results indicated that Ikaros limits splenic B1 cell numbers in the adult in a cell-intrinsic manner, in part by inhibiting their differentiation from bone marrow progenitor cells.

Ikaros-null B1 Cells Are Bona Fide B1 Lineage Cells—To determine if the B1 cells in Ikaros-null mice are *bona fide* B1 cells, we analyzed their transcriptomes. R26 cKO and control littermates were TAM injected and their splenic B1 ($CD19^+CD43^+$) and non-B1 B ($CD19^+CD43^-$) cell populations were analyzed 10d afterward by Affymetrix arrays. Comparison of the mutant and WT B1 cell transcriptomes clearly showed that they were alike in global gene expression (Fig. 4*A*, clusters *a* and *c*, and data not shown). Most of the genes, whose expression levels were previously shown to be increased or decreased in B1a cells compared with FO B cells (43, 44), were similarly increased or decreased in control and R26 cKO B1 cells. Importantly, genes encoding transcription factors essential for B2 cell development and function (*e.g. SpiB*, *Pax5*, *Ebf1*, *Mef2c*) were down-regulated in R26 cKO B1 cells, like control B1 cells. Interestingly, R26 cKO B1 cells expressed intermediate levels of some B2-associated genes (Fig. 4*A*, clusters *b* and *d*), suggesting that Ikaros plays a role in defining the B1 cell signature.

Ikaros deletion resulted in the increase or decrease (fold-change>2) of 96 and 36 genes, respectively, in B1 cells (Fig. 4*B*). Many of the up-regulated genes belonged to the interferon response signature (not shown), which was probably nonspecific and Cre-dependent, as these genes were also up-regulated in R26 cKO thymocytes after TAM treatment, but not in germline-deficient cells (45). In addition, 10 other up-regulated genes corresponded to genes normally expressed in hematopoietic stem cells, which are Ikaros-dependent but also up-regulated in other Ikaros-deficient hematopoietic cells (25). Thus few up-regulated genes appeared to be B cell-specific. Among the B-cell specific genes, some have been implicated in lymphocyte activation, like: *Gna15* (up 2.1×) and *Slamf9* (up 3.5×) (46); *Tmem176b* (up 7.3×) is highly related to the B cell co-signaling molecule CD20 (47); *Nrp2* (up 2×), homologous to *Nrp1*, which is induced in activated T cells (48). In contrast, some of the down-regulated genes are known negative regulators of B cell signaling. These include: *Slpi*, a secretory leukoprotease inhibitor that negatively regulates B cell proliferation and IgM production following LPS activation (down 2.8×) (35); *Tyrobp*, which encodes the Dap12 signaling adaptor, and is a negative regulator of B cell-dependent responses including autoantibody production (down 3×) (49); *Pirb*, which encodes the paired Ig receptor PIR-B, and has been found to negatively regulate BCR-mediated activation and inhibit TLR9-induced autoantibody production (down 2.1×) (50, 51); *Rgs13*, which attenuates G protein-mediated signal transduction and has been shown to limit extrafollicular plasma cell generation (down 2.2×) (52, 53); *Rapgef4*, which encodes Epac, a downstream target of cAMP, and which is implicated in BCR-induced growth arrest and apoptosis (down 2.2×) (54). Many of these changes were validated by RT-qPCR (Fig. 4*C*). The activation of these genes was likely to be Ikaros dependent, as the expression of other transcription factors (*Spi1*, *Pax5*, *Irf4*, *Prdm1*, *Xbp1*, *Tcf1*, *Ebf1*) important for B cell function were similar between Ikaros null and control B1 cells (Fig. 4*D*). To determine if the identified genes were direct Ikaros targets, we analyzed Ikaros binding by ChIP-sequencing on chromatin from a pre-B cell line (22), as primary B1 cell numbers were too limiting for these studies and pre-B cells may contain B1 cell precursors (22) (Fig. 4*E* and data not shown). This showed that Ikaros bound to the loci of *Tyrobp, Nrp2, Tmem176b, Gna15,* and *Slamf9*, suggesting that Ikaros directly regulates the transcription of some of the positive and negative regulator genes. Collectively, these results suggested that Ikaros is required for maintaining the correct expression of regulator molecules of B1 cell activation and signaling.

Ikaros-deficient B1 Cells Hyper-proliferate in Response to TLR4 Stimulation—Because Ikaros has been shown to negatively regulate the activation response in B2 cells (18), we asked if Ikaros also sets the activation threshold in B1 cells, and whether common pathways are regulated by Ikaros in these cell types.

We first compared the proliferative response of splenic B2 FO and B1 cells to stimulation using a variety of stimuli. $Ik^{L/L}$ and WT FO B cells ($CD19^+CD23^{hi}CD21^{lo}$) were stimulated with anti-IgM antibodies, anti-IgM + anti-CD40, anti-CD40 + interleukin-4 (IL-4), or LPS, and cell division was measured by CFSE dilution after 3d of culture. These experiments showed that $Ik^{L/L}$ cells proliferated more (loss of CFSE intensity), and more $Ik^{L/L}$ cells proliferated (sum of the lower CFSE peaks), in response to anti-IgM, anti-IgM + anti-CD40, and anti-CD40 + IL-4 stimulation, when compared with WT cells (Fig. 5*A*), even though cells of both genotypes exhibited similar surface levels of IgM and TLR4 (Fig. 5*B*). However, increased cell division was not detected after LPS stimulation, suggesting that LPS alone saturated the proliferative response in Ikaros-deficient B2 cells. In addition, CD19, CD21, and R26 cKO FO B cells also hyper-proliferated in response to anti-IgM stimulation (Fig. 5*C*). These data extend previous findings, and indicate that FO B2 cells require Ikaros to limit their proliferative response in a B cell-specific and cell-intrinsic manner, especially when stimulation is sub-optimal (*i.e.* anti-IgM stimulation alone). To test if B1 cells also require Ikaros for proliferation, splenic $Ik^{L/L}$ and WT B1 cells ($CD19^+CD43^+$) were stimulated with anti-IgM antibodies or LPS. Although these cells expressed comparable levels of surface IgM and TLR4 (Fig. 5*D*), $Ik^{L/L}$ B1 cells did not respond to anti-IgM stimulation, like WT B1 cells (Fig. 5*E*) (55). However, $Ik^{L/L}$ B1 cells hyper-proliferated to LPS stimulation. Thus, Ikaros sets the activation threshold in B1 cells in response to TLR4 stimulation.

To determine if Ikaros deficiency affects cell survival, we first stimulated $Ik^{L/L}$ and WT FO B cells with anti-IgM ± anti-CD40 antibodies for 18h, and evaluated them with $DiOC_6$, a mitochondria dye that stains live cells (56, 57). This showed that the WT samples contained on average 20% fewer live cells after anti-IgM stimulation (Fig. 5*F*), compared with no stimulation, as expected (58). In contrast, $Ik^{L/L}$ samples stimulated with anti-IgM contained as many live cells as cultures without stimulation, suggesting less BCR-induced apoptosis. Cells of both genotypes showed enhanced viability when stimulated with anti-IgM + anti-CD40, compared with unstimulated cells, and there was little difference between WT and mutant cultures. Thus

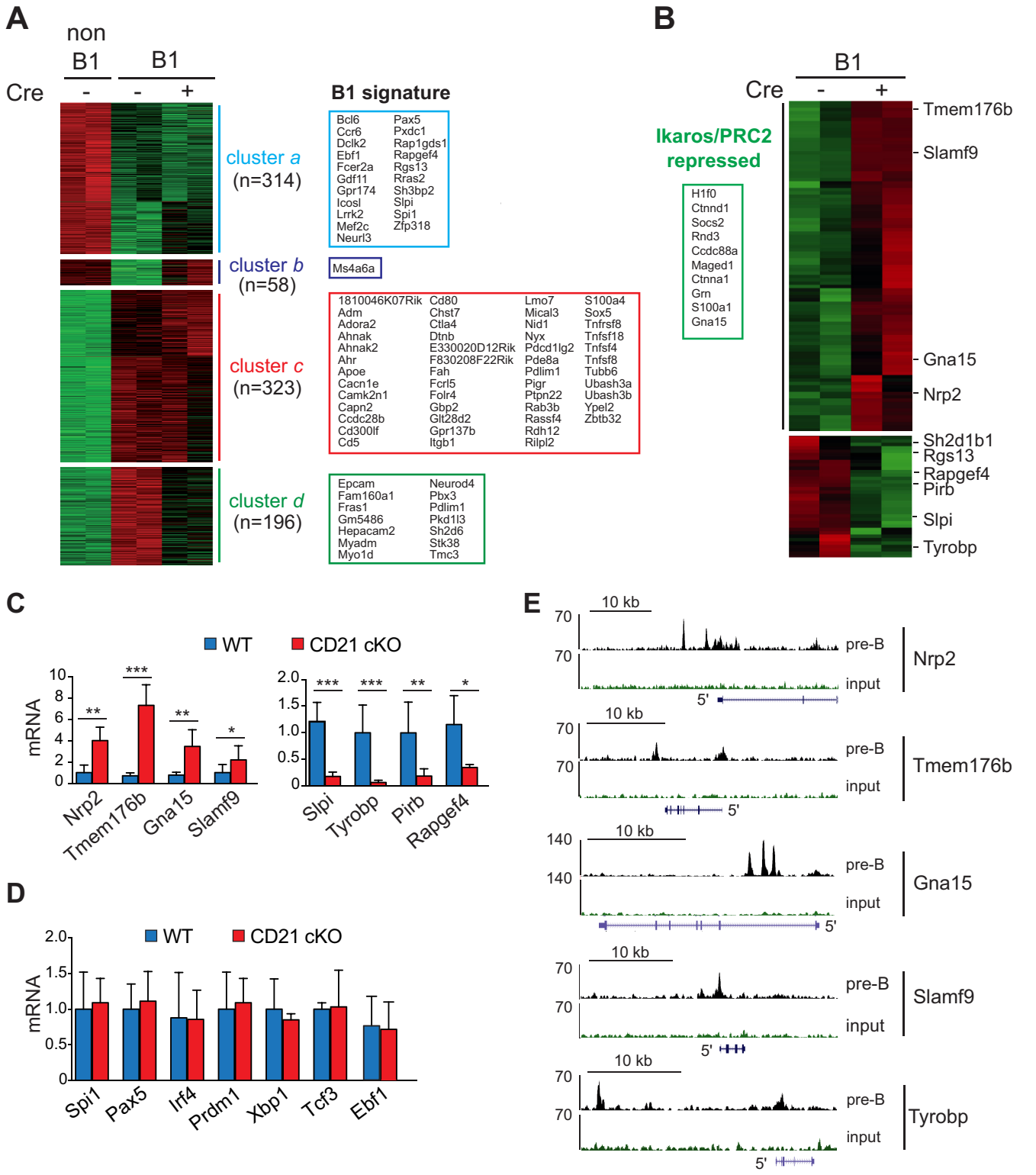


FIGURE 4. **Transcriptome analysis of Ikaros-regulated genes in B1 cells.** *A* and *B*, R26 cKO or control mice were treated with TAM for 3 days. $CD19^+CD43^+$ B1 cells, as well as non-B1 B cells ($CD19^+CD43^-$) were isolated 10d after initiation of TAM treatment, and their transcriptomes were analyzed (two independent samples in each case). *A*, genes up- or down-regulated in the B1 *versus* non-B1 B cells from control mice were identified (log2 FC up or down >0.5 on average, and >0.4 for each pairwise comparison), and the expression of these genes in R26 cKO B1 cells were visualized by K-means clustering. Genes, previously found to be up- or down-regulated in B1 cells (43, 44), are listed next to their respective clusters. *B*, genes up- or downregulated in R26 cKO B1 cells (log2 FC >1, up or down, and >0.8 for each pairwise comparison) were selected and visualized by hierarchical clustering. Up-regulated hematopoietic stem cell genes that were also detected in Ikaros-deficient T cells are listed on the left. Genes that encode positive or negative regulators of signaling are indicated. In *A* and *B*, *green* and *red* colors correspond to low and high values, respectively, of the normalized expression. *C*, RT-qPCR analysis of the indicated genes in WT and CD21 cKO B1 cells relative to HPRT. Graphs represent mean ± S.D. of duplicate samples from 3 mice each. *, $p < 0.01$; **, $p < 0.001$; ***, $p < 0.0001$ (unpaired Student's *t* test). *D*, RT-qPCR analysis of the indicated genes in WT and CD21 cKO B1 cells relative to HPRT. Graphs represent mean ± S.D. of duplicate samples from 3 mice each. *E*, genome browser tracks of Ikaros binding at the loci of the indicated genes in the BH1-Ik1-ER-Bcl2 pre-B cell line (22). Sequencing of input DNA is shown in *green*.

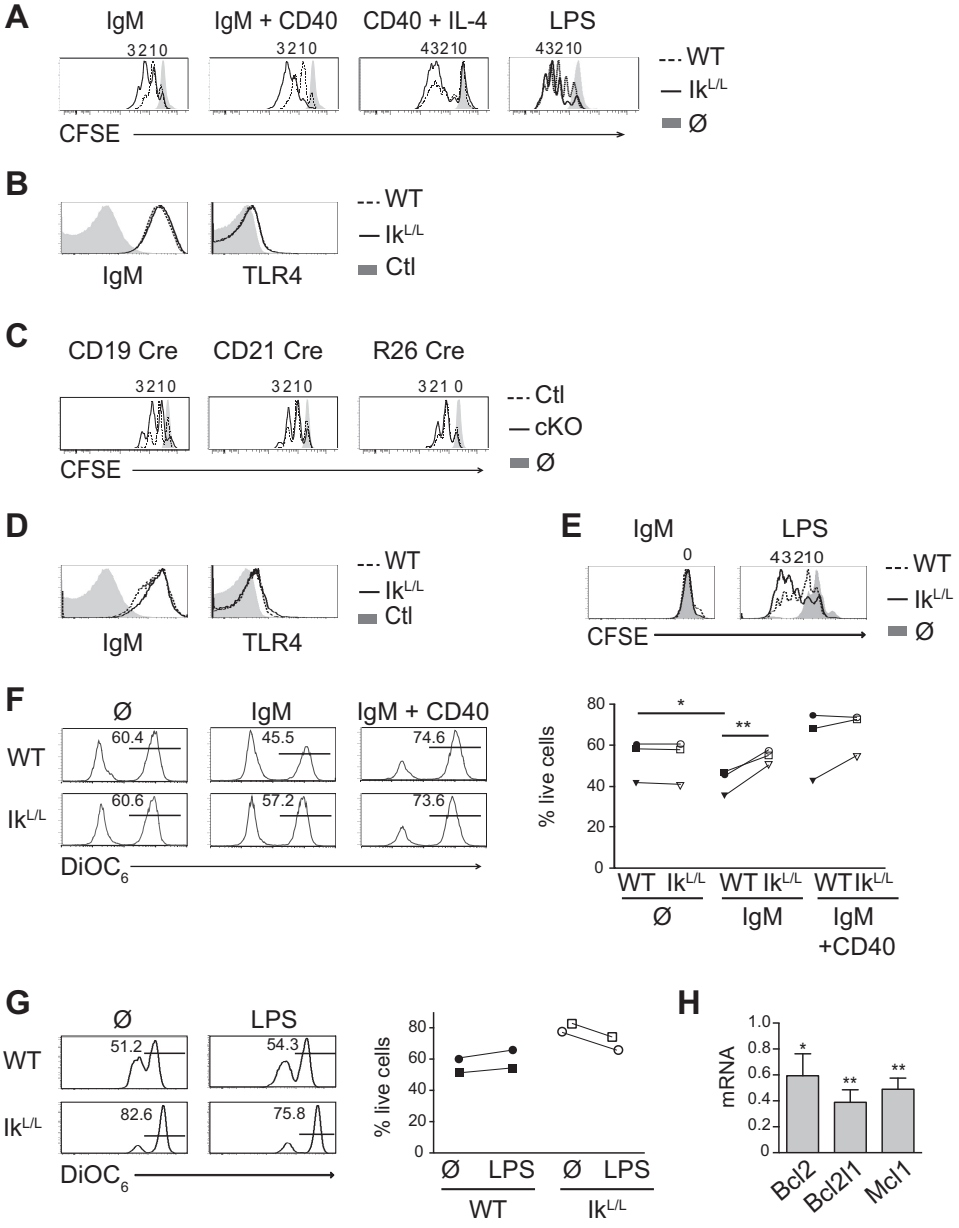
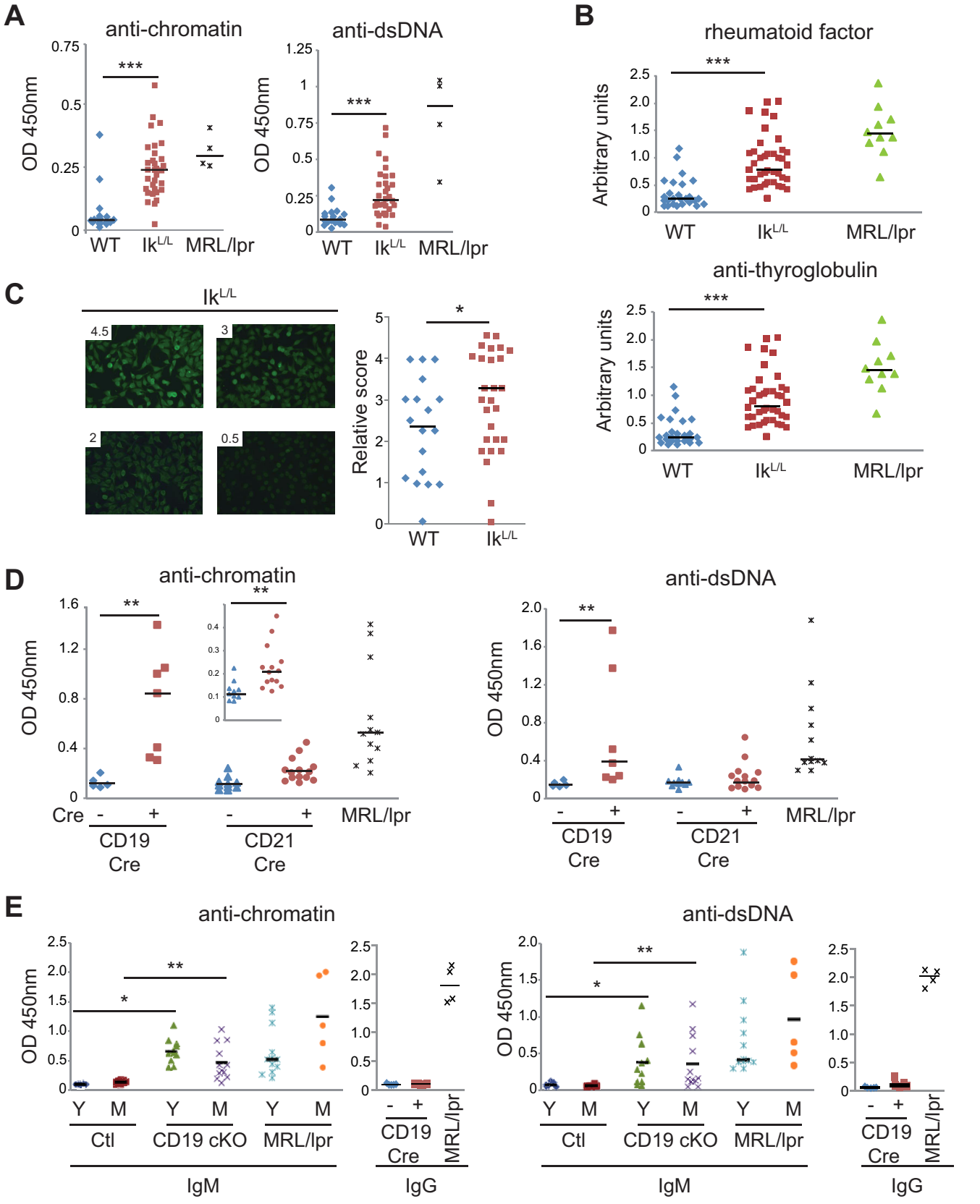


FIGURE 5. **Increased proliferation and survival by Ikaros-deficient B1 cells.** *A*, CFSE-labeled WT and $Ik^{L/L}$ splenic FO B cells were stimulated for 72 h with anti-IgM, anti-IgM + anti-CD40, anti-CD40 + IL-4, or LPS. *Gray histograms* indicate unstimulated WT cells. The number of divisions completed is indicated. Representative of >3 independent experiments. *B*, expression of surface IgM and TLR4 on the indicated FO B cells before stimulation. For IgM, the *gray histogram* denotes non-B cells; for TLR4, the corresponding isotype control. Representative of two independent experiments. *C*, CFSE-labeled splenic FO B cells from the indicated mice were stimulated for 72 h with anti-IgM antibodies. R26 cKO mice were first treated *in vivo* with TAM for 4 days. Ctl, control cells for the corresponding cKO. *Gray histograms* indicate unstimulated cells. Representative of >3 independent experiments for each genotype. *D*, expression of surface IgM and TLR4 on splenic B1 cells before stimulation. For IgM, the *gray histogram* denotes non-B cells; for TLR4, the corresponding isotype control. Representative of two independent experiments. *E*, CFSE-labeled WT and $Ik^{L/L}$ B1 cells were stimulated for 72 h in the presence of anti-IgM or LPS, and analyzed. The number of divisions completed is indicated. *Gray histograms* denote the CFSE labeling for unstimulated cells. Representative of three independent experiments. *F*, WT and $Ik^{L/L}$ FO B cells were stimulated for 18 h with anti-IgM, or anti-IgM + anti-CD40, and analyzed for survival using $DiOC_6$. (*left*) The percentages of the live cells are indicated. (*right*) Statistical analysis of three independent experiments was performed with the indicated samples. *, $p < 0.05$; **, $p < 0.02$ (paired Student's *t* test). *G*, WT and $Ik^{L/L}$ B1 cells were stimulated for 18 h with LPS, then analyzed for survival with $DiOC_6$ to stain active mitochondria (live cells). (*left*) The percentage of live cells is indicated. (*right*) Graph shows quantification of two independent experiments. *H*, relative expression of the indicated genes in unstimulated $Ik^{L/L}$ B1 cells compared with WT (mean ± S.D. of duplicate samples from 3 mice each.). *, $p < 0.01$; **, $p < 0.001$ (unpaired Student's *t* test).

Ikaros-deficient FO B cells survive better than WT cells in response to anti-IgM stimulation. To determine if Ikaros deficient B1 cells also survive better upon stimulation, we stimulated $Ik^{L/L}$ and WT $CD19^+CD43^+$ B1 cells with LPS and evaluated them after 18 h (Fig. 5*G*). Interestingly, in the absence of stimulation, the percentage of live $Ik^{L/L}$ B1 cells was always superior to that of WT cells, even though LPS stimulation decreased $Ik^{L/L}$ cell survival compared with WT B1 cells. Unexpectedly, however, the expression levels of the anti-apoptotic Bcl2 family member proteins (*Bcl2*, *Bcl2l1*, *Mcl1*) were decreased in the mutant B1 cells, suggesting that other mechanisms are behind the increased survival of Ikaros-deficient cells (Fig. 5*H*). Altogether, our results indicated that Ikaros negatively regulates B1 cell survival under non-stimulating conditions.

Ikaros Is a Negative Regulator of Autoantibody Production in B1 Cells—Because B1 cells constitute a major source of self-reactive IgM antibodies *in vivo*, we asked if loss of Ikaros affects the serum autoantibody pool. We previously showed that total IgM antibodies were slightly increased in $Ik^{L/L}$ mice compared with control animals (18). Here we tested the serum of $Ik^{L/L}$ and



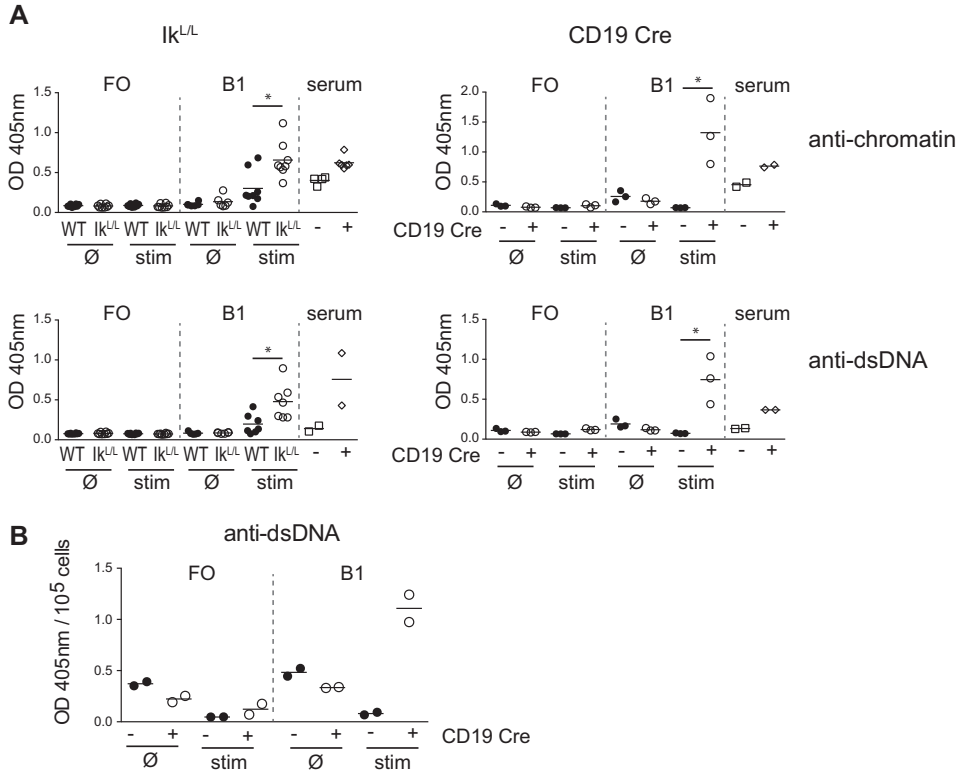


FIGURE 7. **Ikaros-deficient B1 cells secret more anti-chromatin and anti-dsDNA autoantibody *in vitro*.** *A*, splenic FO and B1 cells from $Ik^{L/L}$ and WT mice (*left*), or CD19 cKO (*right*) and control mice were cultured in the absence or presence of anti-IgM + anti-CD40 + LPS for 6 days. Supernatants from the cultures were tested for the indicated antibodies by ELISA. Sera of CD19 cKO (+) and control (−) mice were used as controls. Each *circle* represents data from one mouse. *Graphs* represent data from ≥3 independent experiments. *, $p < 0.01$ (unpaired Student's *t* test). *B*, assessment of anti-dsDNA autoantibodies by ELISA in the supernatants of CD19 cKO splenic FO and B1 B cells cultured for 6 days in the absence or presence (*stim*) of anti-IgM + anti-CD40 + LPS. Data are normalized to cell numbers. Cumulative data from two independent experiments are shown.

WT mice (6–10 weeks of age) for IgM anti-dsDNA, anti-chromatin, anti-thyroglobulin and rheumatoid factor, by ELISA (Fig. 6, *A* and *B*). As control, the serum of lupus-prone MRL/lpr mice was also analyzed. Strikingly, the levels of all IgM autoantibodies tested were increased in the $Ik^{L/L}$ serum. The $Ik^{L/L}$ serum also exhibited increased antinuclear antibody (ANA) activity, as detected on HEp-2 cells (Fig. 6*C*). Indeed, the serum of young (5–9 weeks) and mature (20–41 weeks) CD19 and CD21 cKO mice contained significantly more anti-chromatin and anti-dsDNA IgM antibodies than control mice (Fig. 6, *D* and *E*), and this was particularly striking in the CD19 cKO serum, where autoantibody levels reached those found in the serum of sick MRL/lpr mice. However, the cKO mice did not produce IgG autoantibodies, even at an older age (Fig. 6*E*). These results indicated that Ikaros deficiency in B cells triggers an overproduction of IgM autoantibodies to self-antigens.

We then asked which B cell type was responsible for the autoantibody production. As the CD19 and CD21 cKO mice do not have MZ B cells, and peritoneal B1 cells have not been shown to secrete significant amounts of IgM antibodies (59), we focused our analysis on splenic FO B2 and B1 cells. These cells were sorted from $Ik^{L/L}$ and CD19 cKO (and control) mice, and stimulated with anti-IgM + anti-CD40 + LPS for 6 days, and their supernatants were evaluated for anti-chromatin and anti-dsDNA antibodies by ELISA (Fig. 7*A*). Serum from CD19 cKO and control littermates was used as controls. FO B cells did not secrete detectable autoantibodies, regardless of genotype or stimulation. Likewise, control B1 cells made little or no autoan-

FIGURE 6. **Ikaros deletion enhances anti-chromatin and anti-dsDNA autoantibody secretion *in vivo*.** *A*, sera of $Ik^{L/L}$ ($n = 32$, 6–8 weeks of age) and WT littermates ($n = 18$, 6–9 weeks of age) were tested for IgM antibodies to the indicated antigens by ELISA. MRL/lpr sera ($n = 4$, ~20 weeks of age, diseased) were used as a positive control. *Bars* show the mean of the indicated values. Each *circle* represents one mouse. ***, $p < 0.0001$ (Wilcoxon signed rank test). *B*, sera of $Ik^{L/L}$ ($n = 41$, 6–10 weeks) and WT ($n = 26$, 32–52 weeks) mice were tested for rheumatoid factors (IgM anti-IgG) and anti-Thyroglobulin (IgM) antibodies by ELISA. MRL/lpr serum ($n = 10$, 20 weeks) was used as a positive control. Bars denote the mean values. ***, $p < 0.0001$ (two-tailed Student's *t* test assuming unequal variance). *C*, (*left*) representative ANA levels of $Ik^{L/L}$ serum. Numbers represent the scores. (*right*) Relative ANA levels of $Ik^{L/L}$ *versus* WT sera. Scores were blindly assigned to samples on a scale of 0–5 by four individuals, where 5 is the highest intensity. *Bars* indicate the mean of the scores. *, $p < 0.03$ (Wilcoxon signed rank test). *D*, sera of CD19 cKO ($n = 7$) and control ($n = 5$), and of CD21 cKO ($n = 14$) and control ($n = 10$), mice were tested for IgM antibodies to the indicated antigens by ELISA. Mice were between 7–12 weeks of age. MRL/lpr sera from mice of similar ages ($n = 12$, 10 weeks, non-diseased) were used as a positive control. The *inset* in the *left panel* indicates the same values presented on a smaller scale to highlight the statistical significance. Each value represents the average of two independent ELISA tests. *Bars* show the mean of the indicated values. Each *circle* represents one mouse. **, $p < 0.001$ (Wilcoxon signed rank test). *E*, sera of young and mature CD19 cKO (young, $n = 11$; mature, $n = 12$) and control (*Ctl*) littermates (young, $n = 6$; mature, $n = 10$) mice were tested for IgM (young, *Y* and mature, *M*) and IgG (mature, $n = 5$) antibodies to the indicated antigens by ELISA. Young mice were defined as 5–9 weeks; mature mice as 20–41 weeks of age. MRL/lpr sera (non-diseased: $n = 12$, 10 weeks; diseased: $n = 5$, 20 weeks) were used as positive controls. Each value represents the average of two independent ELISA tests. *Bars* denote the mean values. *, $p < 0.01$; **, $p < 0.001$ (Wilcoxon signed rank test).

tibodies, even upon stimulation. However, both mutant B1 cells secreted significant quantities of IgM anti-chromatin and anti-dsDNA antibodies upon stimulation. Increased antibody secretion was found even after normalizing for cell numbers (Fig. 7*B*). These results thus identify splenic B1 cells as a major source of autoantibody production in Ikaros-deficient mice.

Discussion

Our results indicate that Ikaros is a major negative regulator of B1 cell development and function. Upon loss of Ikaros, B1 cell numbers are increased in the spleen and bone marrow, and adult B1 progenitors are enhanced in the bone marrow. Ikaros-null B1 cells resemble WT B1 cells at the transcriptome level and in their ability to respond to TLR4 stimulation but not anti-IgM activation. However, mutant B1 cells are hyper-reactive, and only Ikaros-null B1 cells can be induced to secrete high amounts of IgM autoantibodies. These results provide a possible explanation for why dominant-negative Ikaros proteins were previously associated with autoantibody production (60).

At least part of the Ikaros null B1 cell population develops from the bone marrow, as Ikaros deficiency in the germline ($Ik^{L/L}$, R26 cKO) or bone marrow (CD19 cKO) leads to an increase in BM B1 cell numbers, while loss of Ikaros in the transitional B cells of the spleen (CD21 cKO) does not. Why CD21 cKO mice also have increased splenic B1 cell numbers remains unknown, although this may be connected to the increase in cell survival of Ikaros-deficient cells that we observed in culture (see Fig. 5). Our results thus support the concept that B1 cells can develop from the bone marrow in addition to the fetal liver, and suggest that WT BM B1 cell development is normally kept in check by Ikaros. This is in line with our previous observations that $Ik^{L/L}$ fetuses show a near-complete loss of B lineage cells (18). Interestingly, PEC B1 cells do not appear to be negatively regulated by Ikaros in the mouse lines studied here, suggesting different or additional mechanisms of regulation.

Several lines of evidence suggest that Ikaros null B1 cells develop from B1 progenitor cells and not from committed B2 cells. At the transcriptional level, the mutant B1 cells exhibit a gene expression signature most resembling WT B1 cells. At the functional level, Ikaros-deficient B1 cells behave like WT B1 cells, as they do not proliferate in response to anti-IgM stimulation and they do not undergo class switch recombination to secrete IgG autoantibodies. Further, we were unable to differentiate B1 cells from 4OHT-treated R26 cKO spleen cells (BH, unpublished results) like we did from the BM, arguing against a possible switch from B2 to B1 cells in the spleen, as has been reported for PU.1-null B cells (11). Thus, although Ikaros sets the activation threshold for both B2 and B1 cells, this does not appear to influence the cell surface and functional phenotype of B1 cells.

Nonetheless, Ikaros-deficient B1 cells show downregulated mRNA levels for important negative regulators of B cell signaling and autoantibody production. These genes may be direct Ikaros targets, as Ikaros binds to the loci of several of these genes in pre-B cells. Further, we found that many transcriptional regulators essential for B2 and B1 cell biology remain relatively unchanged between control and Ikaros-null B1 cells, suggesting that they do not play a major role in regulating the activation threshold or state of Ikaros null B1 cells. This is in contrast to a recent study in peritoneal B1 cells in which the knockdown of Ikaros by siRNA induced an up-regulation of *Spi1* mRNA levels (61), suggesting perhaps that peritoneal and splenic B1 cells respond differently to Ikaros.

Our results indicate that Ikaros is an important transcriptional activator in B1 cells, similar to what we and others have observed in pre-B cells (22, 23). This is in contrast to T cells where Ikaros deficiency leads to the activation of the Notch pathway and hematopoietic stem cell-associated genes (25, 45), indicating a key role as a transcriptional repressor. Previous studies of transcriptional regulation in B1 cells have mainly identified proteins important for promoting B1 cell function. Ikaros, on the other hand, appears to be unique in that it is required to limit B1 cell development, survival, and expansion.

Finally, our results have implications for Ikaros and B1 cells in autoimmunity and cancer. B1 cells and polyreactive antibodies are elevated in several autoimmune diseases, and the *IKZF1* gene was identified as a susceptibility locus for systemic lupus erythematosus (62, 63). Further investigation into the development and function of B1-like cells from patients with *IKZF1* polymorphisms may reveal a similar deregulation of B1 cell function in the human system. Similarly, Ikaros is a tumor suppressor in B cell acute lymphoblastic leukemias (B-ALL) (64), particularly those with oncogenic mutations or amplifications that activate the JAK-STAT pathway (*e.g.* BCR-ABL, JAK2, CRLF2). Many cases debut in early childhood, a time when human B1 cells have been speculated to be abundant (65). Indeed, BCR-ABL transformed murine B1 progenitor cells initiate disease more rapidly than B2 progenitors, suggesting that B1 cells may be involved in high risk cases (66). Our results may therefore suggest a crucial link between Ikaros loss and B1 cell-associated ALL. Finally, as $CD5^+$ B1 cells have been implicated in chronic lymphoid leukemia (CLL) (67), Ikaros may also act as a tumor suppressor in B1 cell-related CLL.

Author Contributions—A. M.-G., B. H., M. S., P. K., and S. C. designed and performed the research, analyzed data, and wrote the paper. P. M. generated and analyzed mutant mice. B. H., H. D., S. M. performed and analyzed ELISAs (chromatin, dsDNA) and immunofluorescence experiments. J. L. P. analyzed some of the ELISAs (RF, thyroglobulin). S. M. and J. L. P. contributed to scientific discussions.

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